Attorney Docket No.: IMGNTX.001US

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Applicant	:	William P. Spencer, et al.
Appl. No.	:	10/805,386
Filed	:	March 22, 2004
Title	:	ESTERIFIED FATTY ACID COMPOSITION
Examiner	:	D. Carr
Group Art Unit	:	1621
Conf. No.	:	1731

DECLARATION OF HATICE HASTURK
SUBMITTED UNDER 37 C.F.R. § 1.132

I, Hatice Hasturk, D.D.S., Ph.D., declare as follows:

1. I am an assistant professor at the Department of Periodontology and Oral Biology, Boston University Goldman School of Dental Medicine, where I am also a research coordinator at the Clinical Research Center.

2. I received my MSc in Dentistry and my D.D.S. (1988) from University of Hacettepe (Ankara-Turkey) Faculty of Dentistry, and my Ph.D. (1995) from University of Hacettepe (Ankara-Turkey), Faculty of Dentistry, Department of Periodontology. I received a Certificate in Advanced Graduate Studies (CAGS) in Periodontology (2004) from Boston University Goldman School of Dental Medicine. I was a Research Associate (Full Time) at Hacettepe University, Faculty of Dentistry, Department of Periodontology from 1988-1995; a practicing periodontist at Cosmodent Dentistry and Implantology Center, Istanbul, TURKEY, from 1995-1999; Postdoctoral Research Associate at Clinical Research Center, Boston University Goldman School of Dental Medicine, Department of Periodontology & Oral Biology from 1999-2002; and Research Coordinator at the Clinical Research Center (2000-present) and Assistant Professor at the Department of Periodontology & Oral Biology (2002-present) at Boston University Goldman School of Dental Medicine. My research has been funded by various funding agencies, such as the National Institutes of Health and the National Science Foundation. I have over 25 publications in peer-reviewed journals and have made close to 75 presentations at scientific meetings.

3. I have read and understood the specification of the above-captioned patent application, the currently pending claims as submitted to the U.S. Patent and Trademark Office (PTO) on January 8, 2008, and the most recent Office Action, mailed by the PTO on April 4, 2008.

4. Pursuant to a grant from Imagenetix, Inc., the assignee of the above-captioned patent application, I studied the actions of various cetylated and non-cetylated fatty acid compounds on monocyte-mediated cytokine response. 1-Tetradecanol complex (1-TDC) is a monounsaturated fatty acid mixture, which contains a blend of cetylated monounsaturated fatty acids. The aim of my study was to describe the anti-inflammatory actions of 1–TDC and various cetylated fatty acids used in this complex using monocytes/macrophages in vitro. In an effort to determine the actions of cetylated fatty acids, various non-cetylated fatty acids were also tested.

5. The study was approved by the Boston University Medical Center Institutional Review Board (BUMC IRB). Peripheral blood was obtained from medically healthy volunteers without periodontal disease and with no known medication use after obtaining signed informed consent. For these experiments, peripheral blood mononuclear cells were isolated by Ficoll-Hypaque density gradient centrifugation. Mononuclear phagocytes (=monocytes) were separated from other mononuclear cells (e.g. lymphocytes) by adherence over 2 hours and pure cell cultures were treated with various doses of tested compounds for 30 minutes. Cells were incubated with 1-TDC (obtained from Imagenetix, Inc.), 2-TDC, which has the non-cetylated forms of the monounsaturated fatty acids found in 1-TDC, myristic acid, palmityl oleate, palmityl myristoleate, palmityl myristate, and cetyl myristate (obtained from Sigma Aldrich) in different concentrations (10^{-9} to 10^{-5} M). Vehicle (5% ethyl alcohol and 5% methyl beta cyclodextrin), which was used to dissolve the compounds into an aqueous preparation, was used as the negative control while dexamethasone (1 nM) was used as the positive control. After incubation with test compounds, half the samples were treated with lipopoylsaccharides (LPS) from *E. coli* (100 ng/mL) as the activator of cell cytokine release over various time points (6, 24, and 48 hours) at 37 °C under 5% CO_2 while the other half served as internal control and were not treated with LPS. Supernatants were collected at 6, 24, and 48 hours and were stored at –80 °C until analyzed. Each sample was prepared in triplicates. The cytokine release was analyzed by xMAP multiplexing technology using Luminex 100 platform. This method allowed us simultaneous analyses of all the cytokines proposed in this experiment. The data was presented as %-inhibition over the vehicle's effect of LPS-mediated cell activation. At least 10% inhibition is considered as inhibitory effect to demonstrate the potential impact of these compounds.

6. Inhibition potential of various compounds on monocyte-mediated cytokine release tested at different concentrations is shown in tables below. Table 1 shows that cetyl myristate inhibits TNF-α as early as 6 hours. IL-8, which is a strong chemoattractant and mainly released by neutrophils, was also detected at high levels at 6 hours and was significantly inhibited by all compounds tested. MCP-1, which is also a strong chemokine for monocytes, was inhibited by 1-TDC and cetyl myristate. None of the other tested compounds were strong inhibitors of the monocyte-mediated cytokine release at 6 hours.

Table 1: Inhibition of cytokine release from peripheral blood monocytes over 6 hours.

Compound	*TNF-α*	IL-1β	IL-6	IL-12	IL-8	MCP-1
1-TDC					√	√
2-TDC					√	
Cetyl Myristate	√				√	√
Myristic Acid					√	
Palmityl Myristoleate					√	

7. Table 2 demonstrates the 24-hour inhibitory potential of the tested compounds. At this time point, palmityl oleate and palmityl myristoleate significantly and fully inhibited both the TNF-α and IL-1β release. Cetyl myristate's inhibition on TNF-α continued at 24-hour where a significant inhibition was seen. 1-TDC, 2-TDC, and palmityl myristate also blocked TNF-α release, however the inhibition was weaker compared to cetyl myristate. In addition to TNF-α, cetyl myristate also inhibited IL-8 and MCP-1 release. While all tested compounds inhibited IL-8 release, 2-TDC also blocked IL-6, which is a potent pro-inflammatory cytokine release by monocytes and MCP-1. Myristic acid, while failed to inhibit the TNF-α release, significantly blocked all other compounds tested at 24-hour.

Table 2: Inhibition of cytokine release from peripheral blood monocytes over 24 hours.

Compound	TNF-α	IL-1β	IL-6	IL-12	IL-8	MCP-1
1-TDC	√				√	
2-TDC	√		√		√	√
Cetyl Myristate	√				√	√
Myristic Acid		√	√	√	√	√
Palmityl Myristate	√				√	
Palmityl Oleate	√	√			√	√
Palmityl Myristoleate	√	√			√	√

8. The figures referenced herein are attached at the end of this declaration, after the signature page. Figure 1 demonstrates the 6-hour-response for all mediators detected and Figures 2-7 show the 24-hour inhibition profiles of each compound and their effective concentrations on the same inflammatory mediators. The 48-hour results demonstrated that the inhibitory potentials of all the compounds detected at 24-hour declined or diminished at the end

of the observation period. Figures 8-13 demonstrate these actions at 48-hour. The 24-hour results are discussed further below.

9. Figure 2 shows the 24-hour inhibition profiles for the inhibition of monocyte-mediated TNF-α release. Only palmityl oleate (PO) and palmityl myristoleate (PMO) show 100% inhibition by this time. Cetyl myristate (CM) also shows significant inhibition. The rest of the compounds show less than 40% inhibition. Myristic acid (MA) shows no inhibitory effect.

10. Figure 3 shows the 24-hour inhibition profiles for the inhibition of monocyte-mediated IL-1β release. Again, palmityl oleate (PO) and palmityl myristoleate (PMO) show 100% inhibition by this time. Myristic acid, which showed no inhibitory effect for TNF-α release, shows significant inhibition of IL-1β release. The rest of the compounds, some of which showed TNF-α release inhibition up to 30%, show no inhibitor effect on IL-1β release.

11. Figure 4 shows the 24-hour inhibition profiles for the inhibition of monocyte-mediated IL-6 release. With this mediator, myristic acid (MA) shows 100% inhibition. 2-TDC, which was inactive with respect to TNF-α and IL-1β, shows about 50% inhibition. 1-TDC, also inactive with respect to TNF-α and IL-1β, shows some activity. The rest of the compounds are completely inactive, even though some of them showed activity with respect to TNF-α and IL-1β.

12. Figure 5 shows the 24-hour inhibition profiles for the inhibition of monocyte-mediated IL-12 release. Only myristic acid (MA) shows activity. The rest of the compounds are inactive.

13. Figure 6 shows the 24-hour inhibition profiles for the inhibition of monocyte-mediated IL-8 release. All of the compounds show some degree of activity. Cetyl myristate (CM) and palmityl myristate (PM) are the most active, whereas palmityl oleate (PO) and palmityl myristoleate (PMO) are the least active.

14. Figure 7 shows the 24-hour inhibition profiles for the inhibition of monocyte-mediated MCP-1 release. 1-TDC shows no activity at all and palmityl myristate (PM) shows very little activity. The activities of the rest of the compounds are varied.

15. The data indicate that different compounds have different inhibitory effect on different inflammation mediators. A good inhibitor of one inflammation mediator may not have any effect at all on other inflammation mediators. For example, cetyl myristate (CM) inhibits IL-8 80% by 24 hours (Fig. 6), shows some activity on the inhibition of TNF-α (Fig. 2) and MCP-1 (Fig. 7), but has no effect whatsoever on IL-1β (Fig. 3), IL-6 (Fig. 4), or IL-12 (Fig. 5).

16. Based on the results of these experiments, it is my opinion that positive results from inhibitory effects of one compounds in one inflammation pathway cannot be generalized to assume all anti-inflammatory compounds will be effective in all inflammation pathways. In other words, the effects of anti-inflammatory compounds in a particular inflammation pathway cannot be determined *a priori*. To determine whether a particular compound is effective in a

particular inflammation pathway, the effect of that particular compound in that particular inflammation pathway should be studied experimentally.

17. I declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful, false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful, false statements may jeopardize the validity of the application or patent issuing therefrom.

Dated: Nov 26, 2008 By: _____
Hatice Hasturk, D.D.S., Ph.D.

Figure 1 Inhibition of monocyte-mediated cytokine release (6 hours)

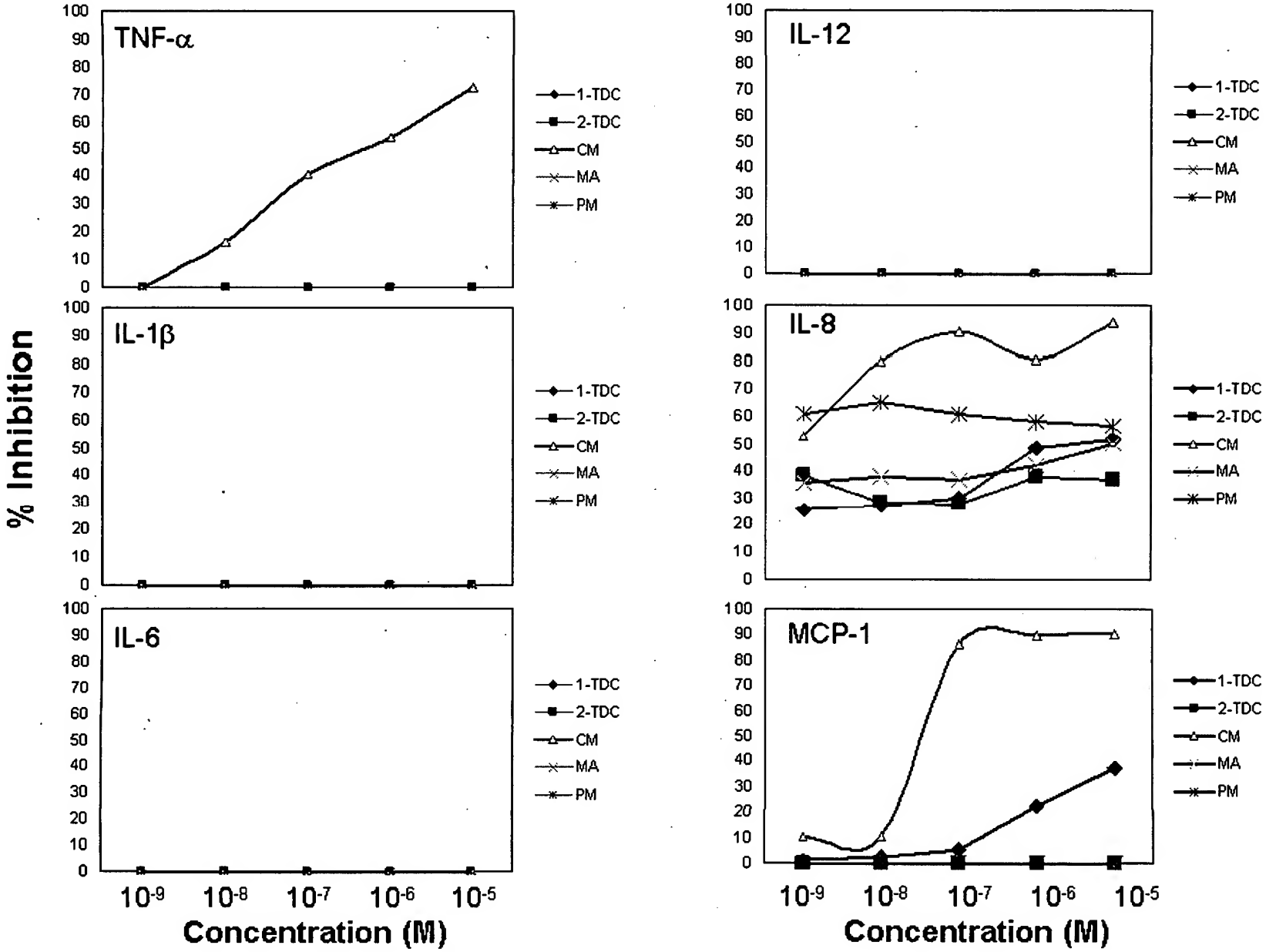


Figure 2

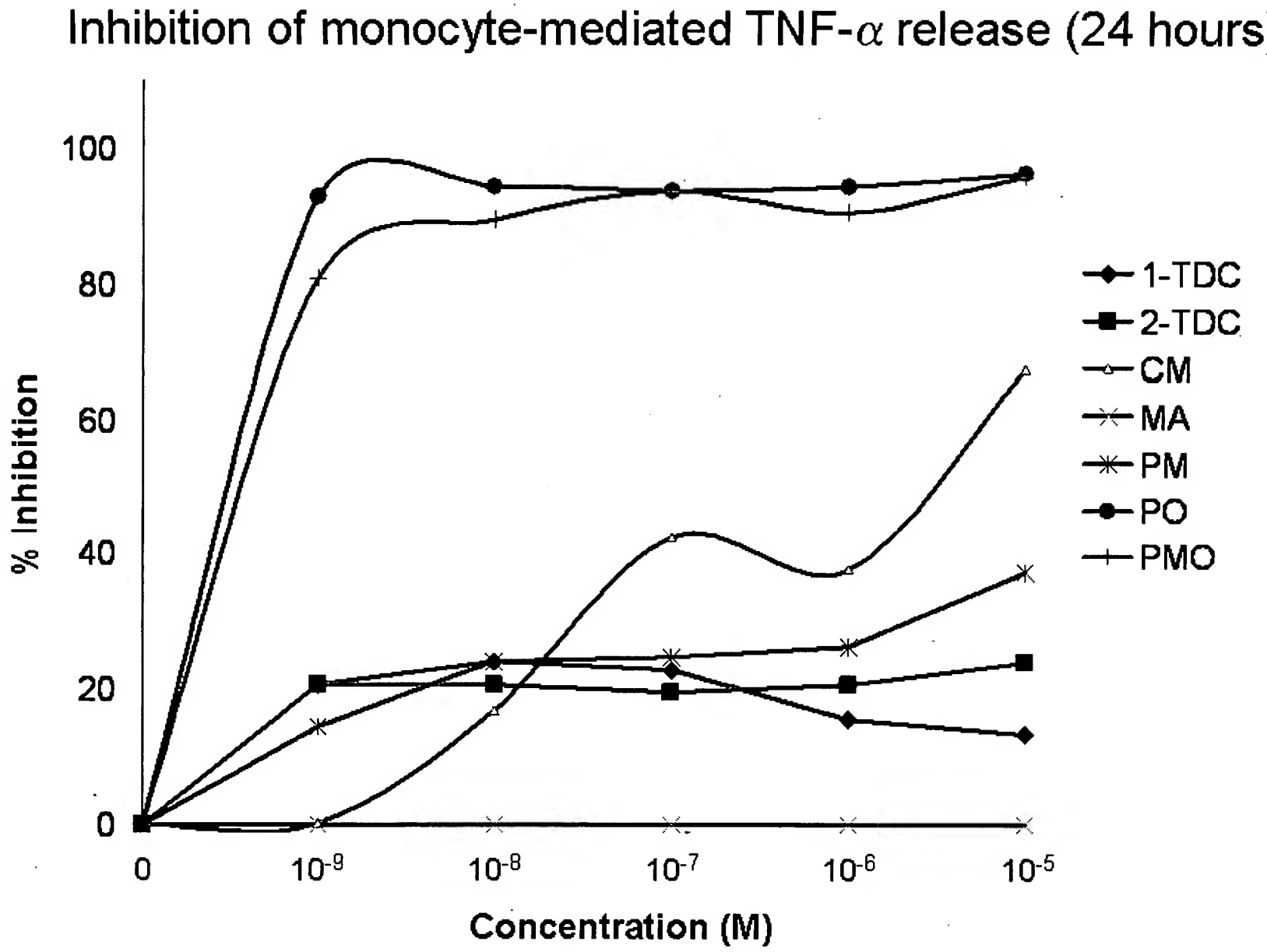

Inhibition of monocyte-mediated TNF-α release (24 hours)
% Inhibition
100
80
60
40
20
0
0
10-9
10-8
10-7
10-6
10-5
Concentration (M)
1-TDC
2-TDC
CM
MA
PM
PO
PMO

Figure 3

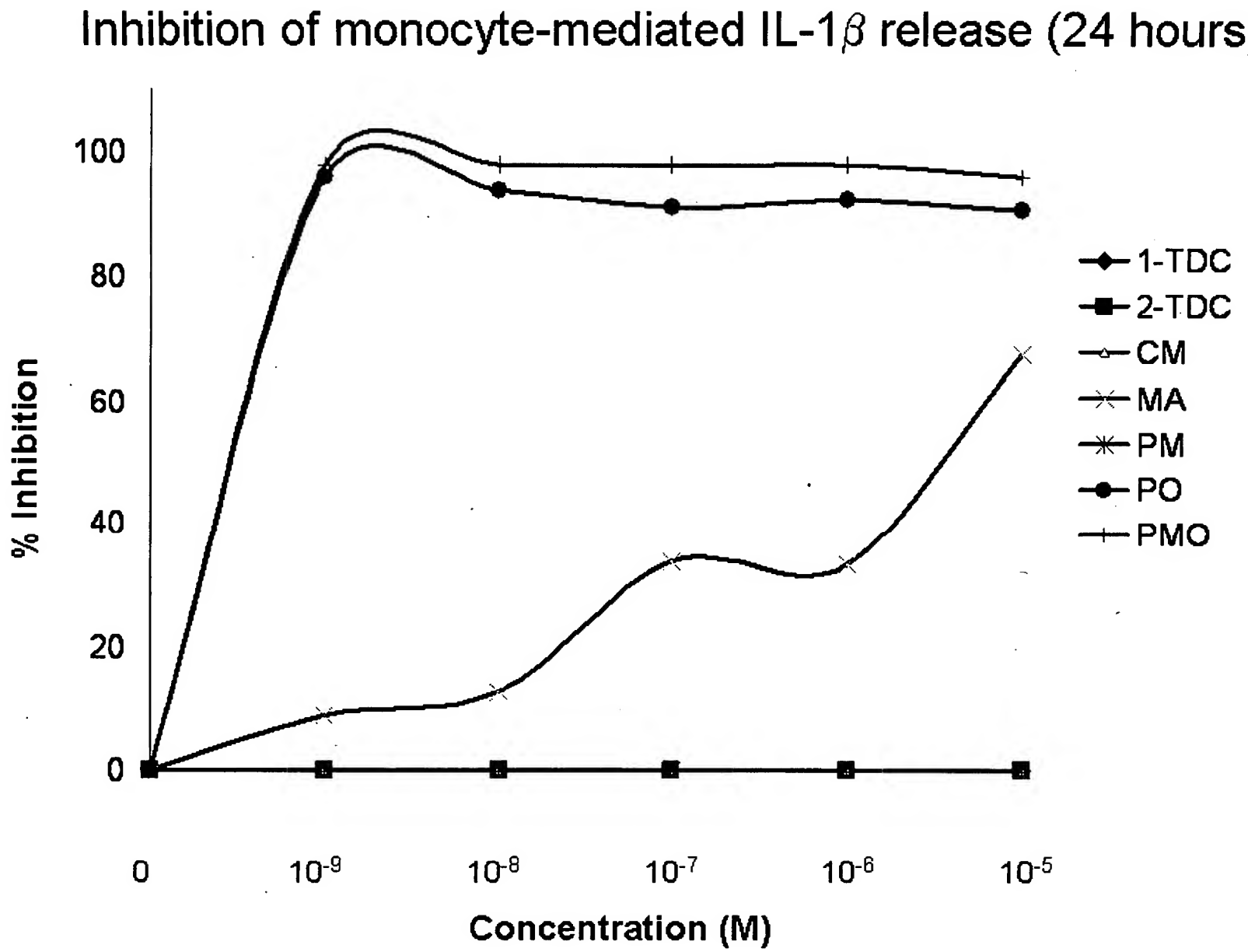

Inhibition of monocyte-mediated IL-1β release (24 hours)
% Inhibition
100
80
60
40
20
0
0
10-9
10-8
10-7
10-6
10-5
Concentration (M)
1-TDC
2-TDC
CM
MA
PM
PO
PMO

Figure 4

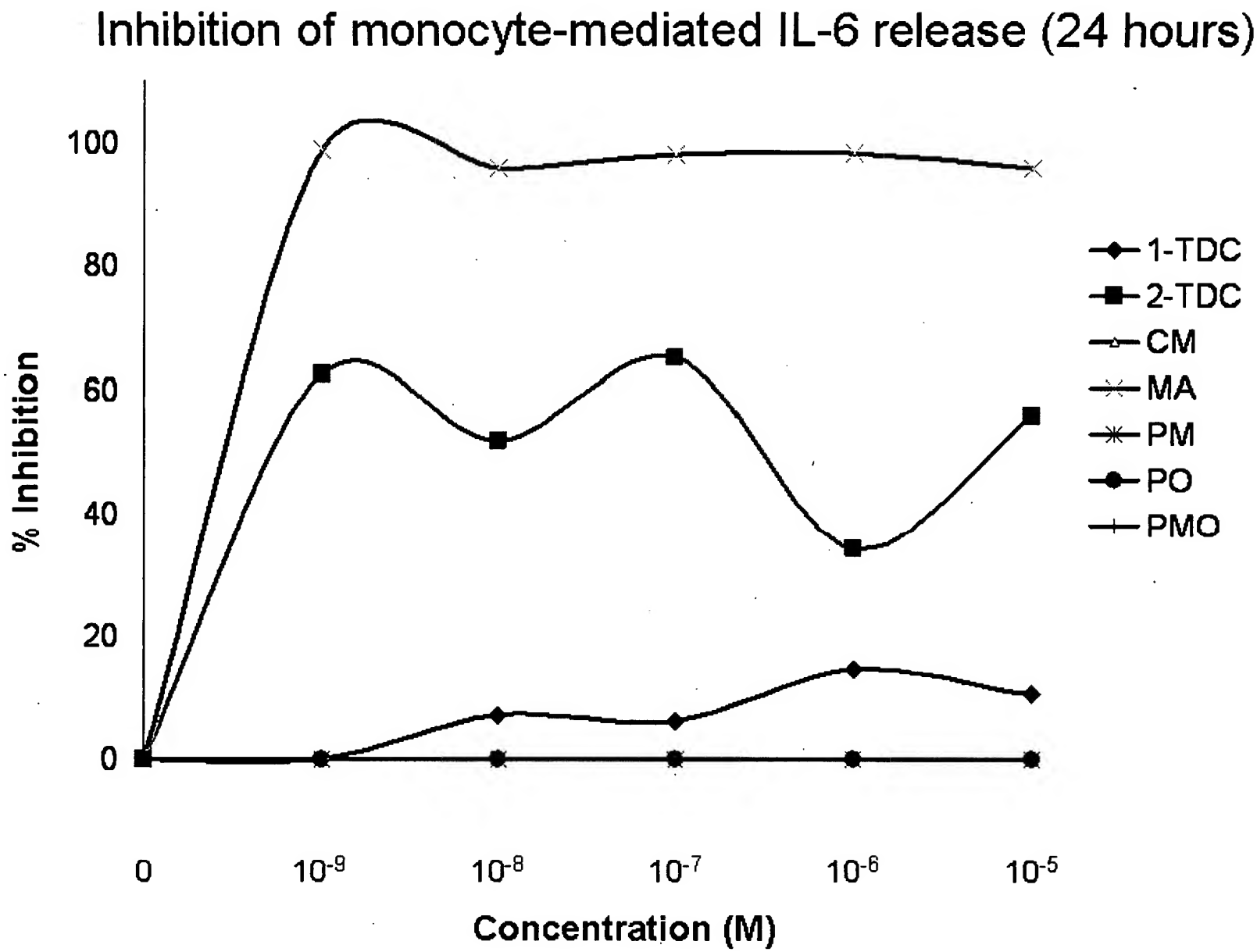

Inhibition of monocyte-mediated IL-6 release (24 hours)
% Inhibition
100
80
60
40
20
0
0
10-9
10-8
10-7
10-6
10-5
Concentration (M)
1-TDC
2-TDC
CM
MA
PM
PO
PMO

Figure 5

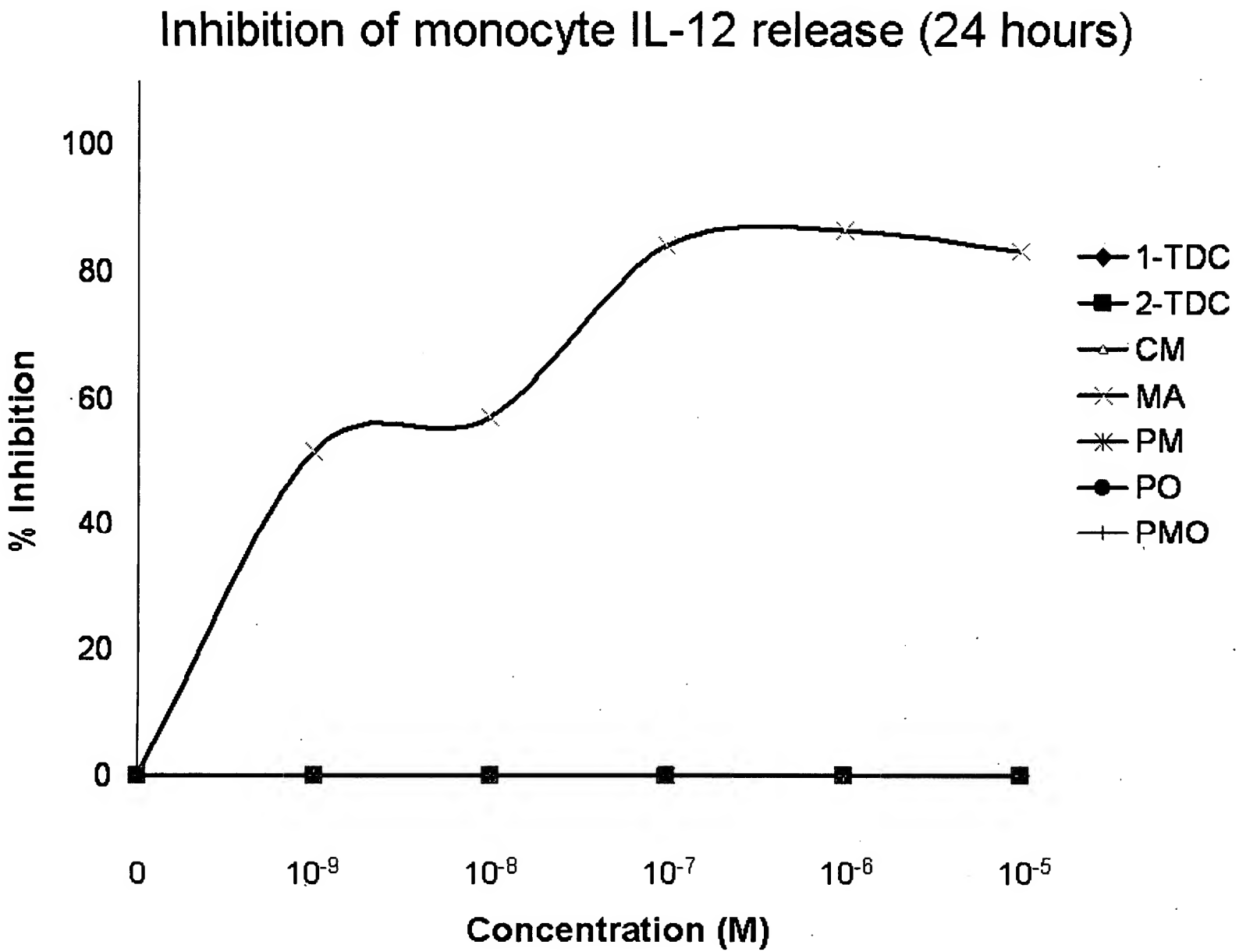

Inhibition of monocyte IL-12 release (24 hours)
% Inhibition
100
80
60
40
20
0
0
10-9
10-8
10-7
10-6
10-5
Concentration (M)
1-TDC
2-TDC
CM
MA
PM
PO
PMO

Figure 6

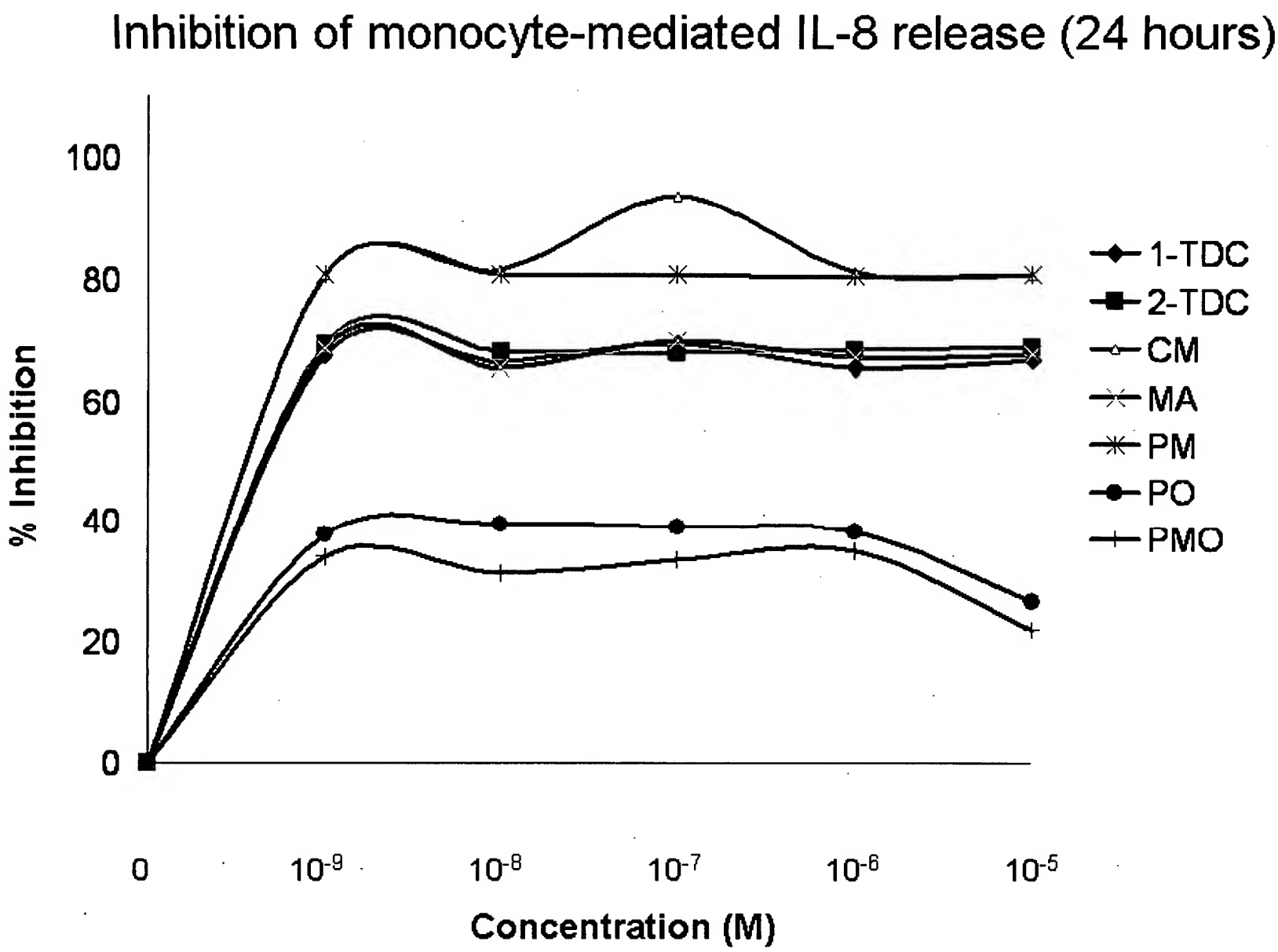

Inhibition of monocyte-mediated IL-8 release (24 hours)
% Inhibition
100
80
60
40
20
0
0
10-9
10-8
10-7
10-6
10-5
Concentration (M)
1-TDC
2-TDC
CM
MA
PM
PO
PMO

Figure 7

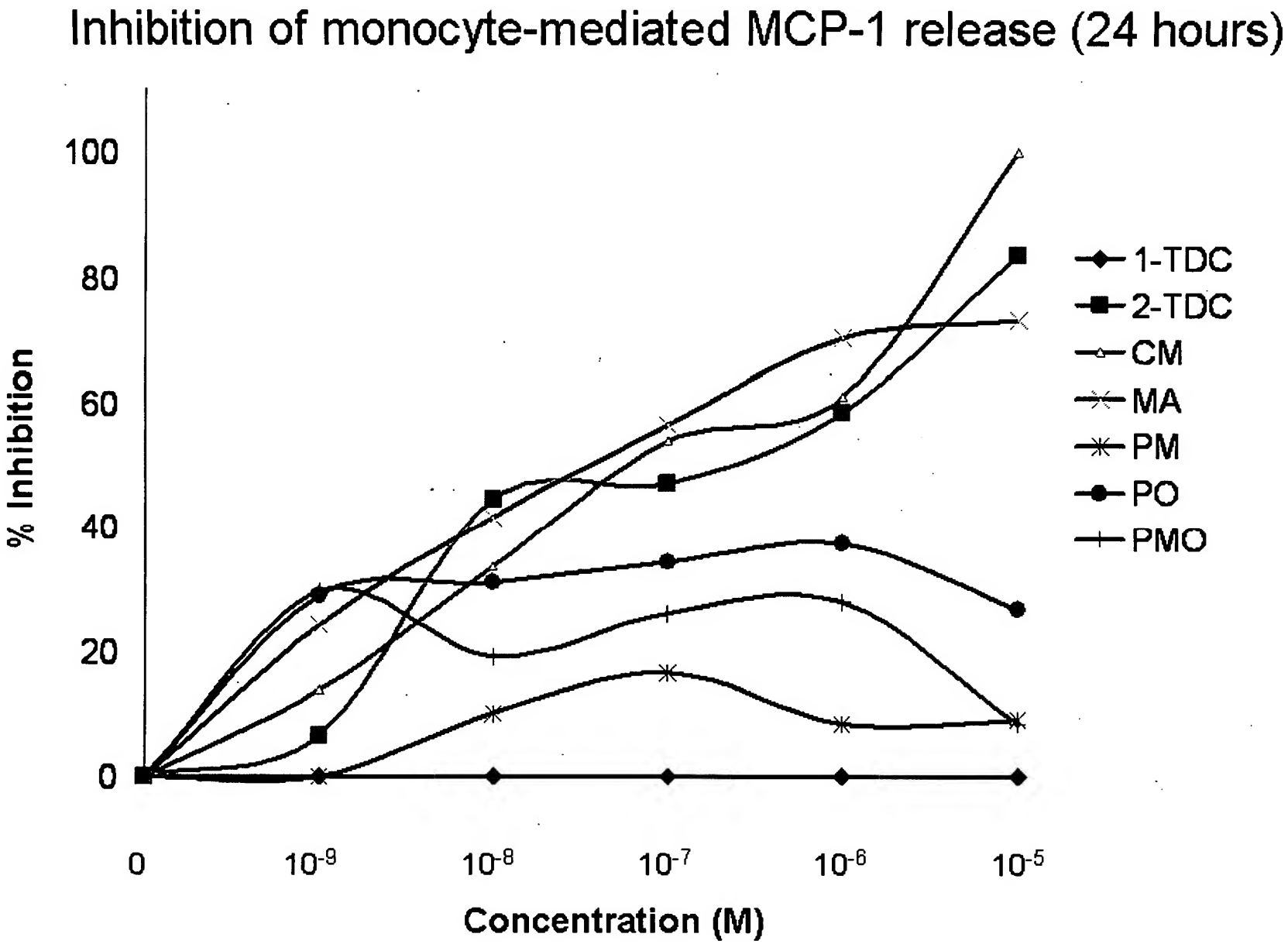

Inhibition of monocyte-mediated MCP-1 release (24 hours)
% Inhibition
100
80
60
40
20
0
0
10-9
10-8
10-7
10-6
10-5
Concentration (M)
1-TDC
2-TDC
CM
MA
PM
PO
PMO

Figure 8

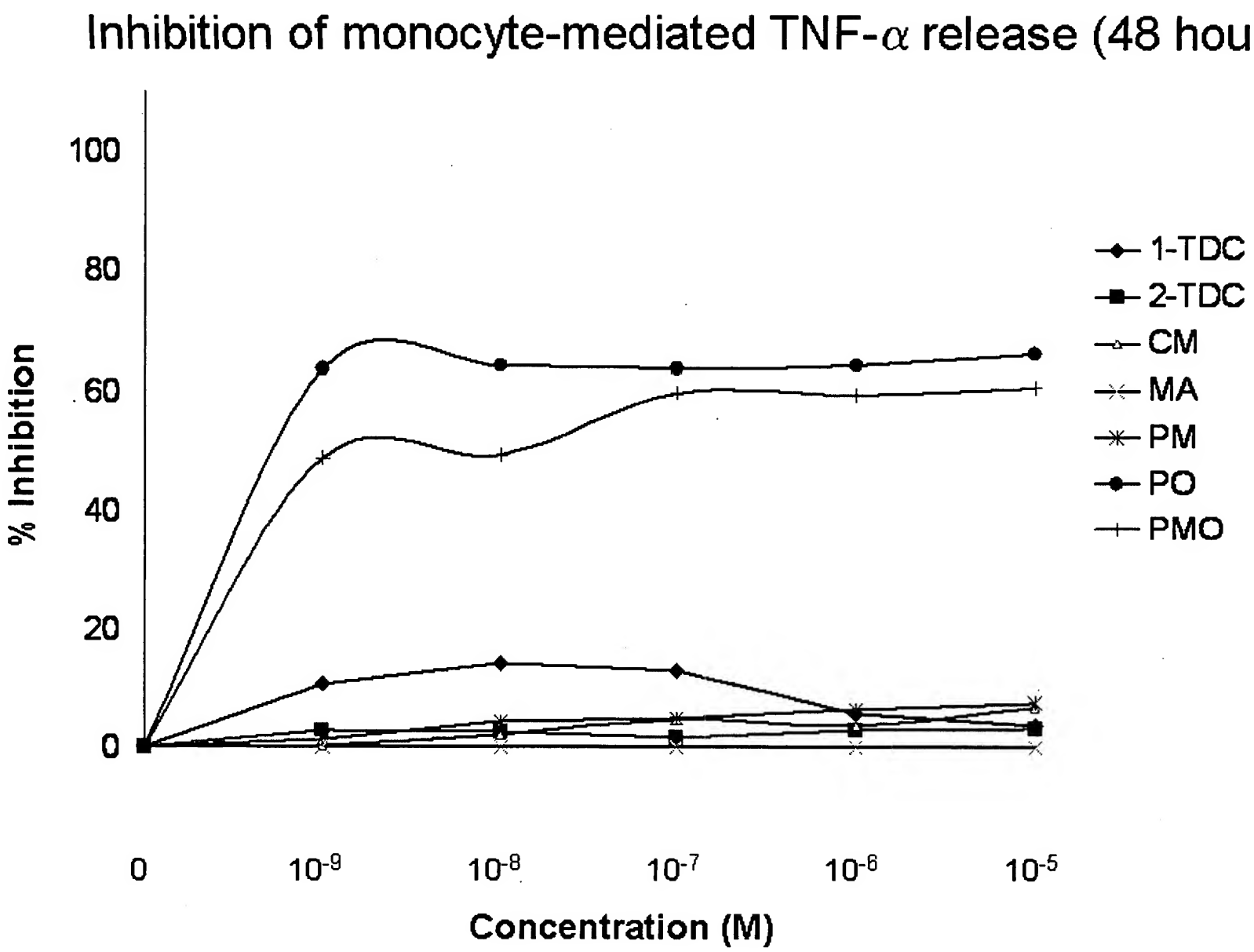

Inhibition of monocyte-mediated TNF-α release (48 hours)
% Inhibition
100
80
60
40
20
0
0
10-9
10-8
10-7
10-6
10-5
Concentration (M)
1-TDC
2-TDC
CM
MA
PM
PO
PMO

Figure 9

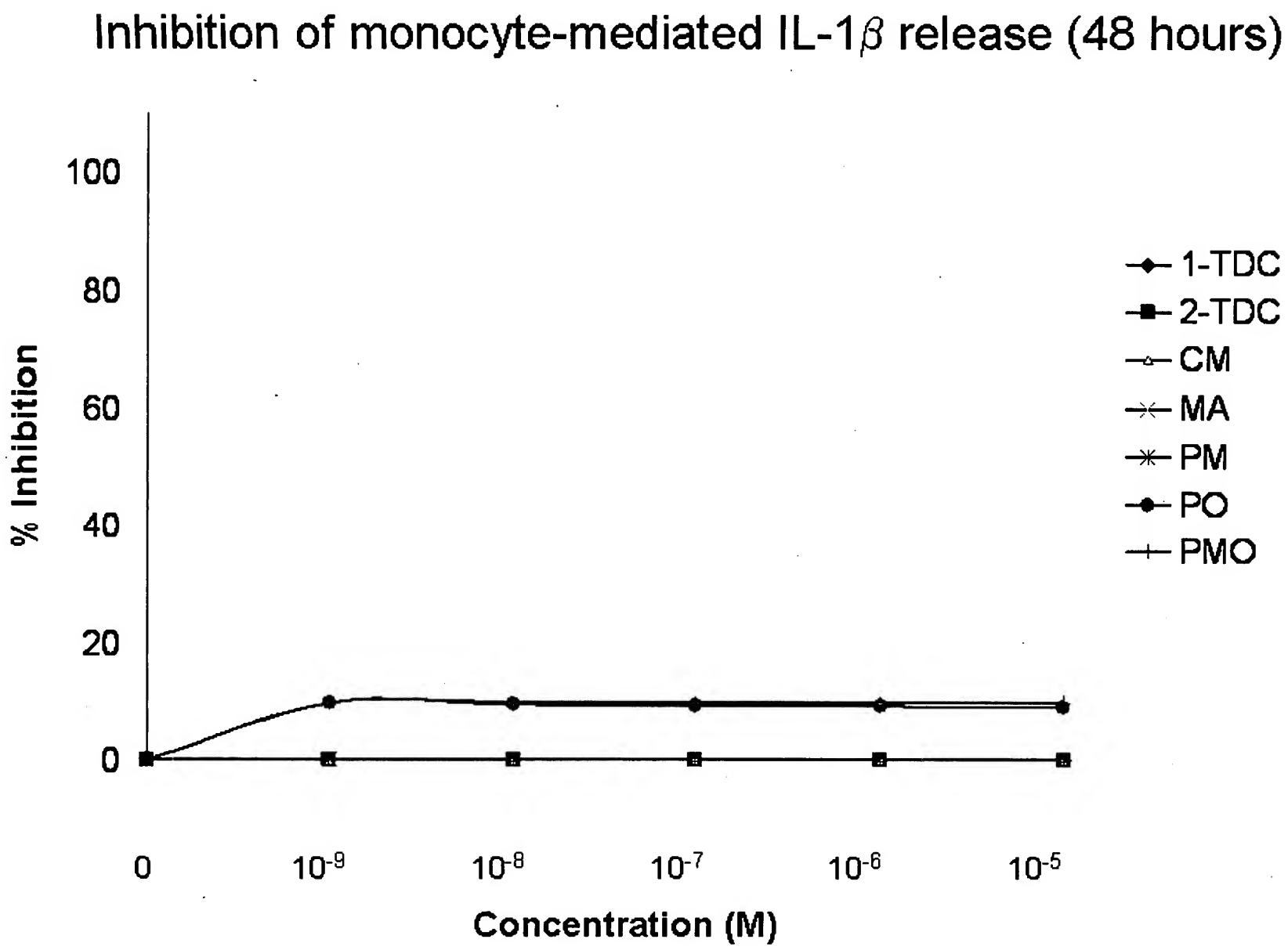

Inhibition of monocyte-mediated IL-1β release (48 hours)
% Inhibition
100
80
60
40
20
0
0
10-9
10-8
10-7
10-6
10-5
Concentration (M)
1-TDC
2-TDC
CM
MA
PM
PO
PMO

Figure 10

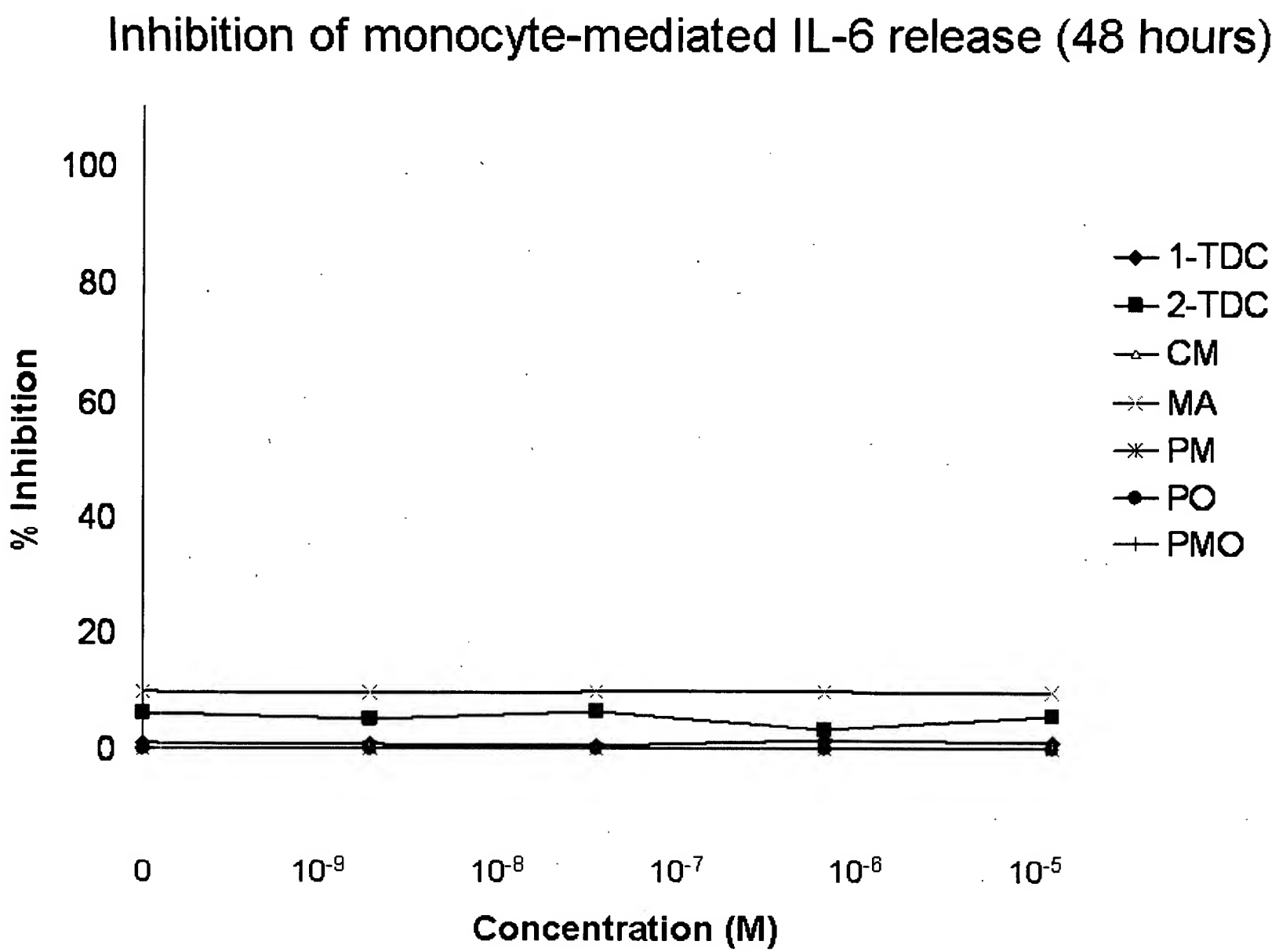

Inhibition of monocyte-mediated IL-6 release (48 hours)
% Inhibition
100
80
60
40
20
0
0
10-9
10-8
10-7
10-6
10-5
Concentration (M)
1-TDC
2-TDC
CM
MA
PM
PO
PMO

Figure 11

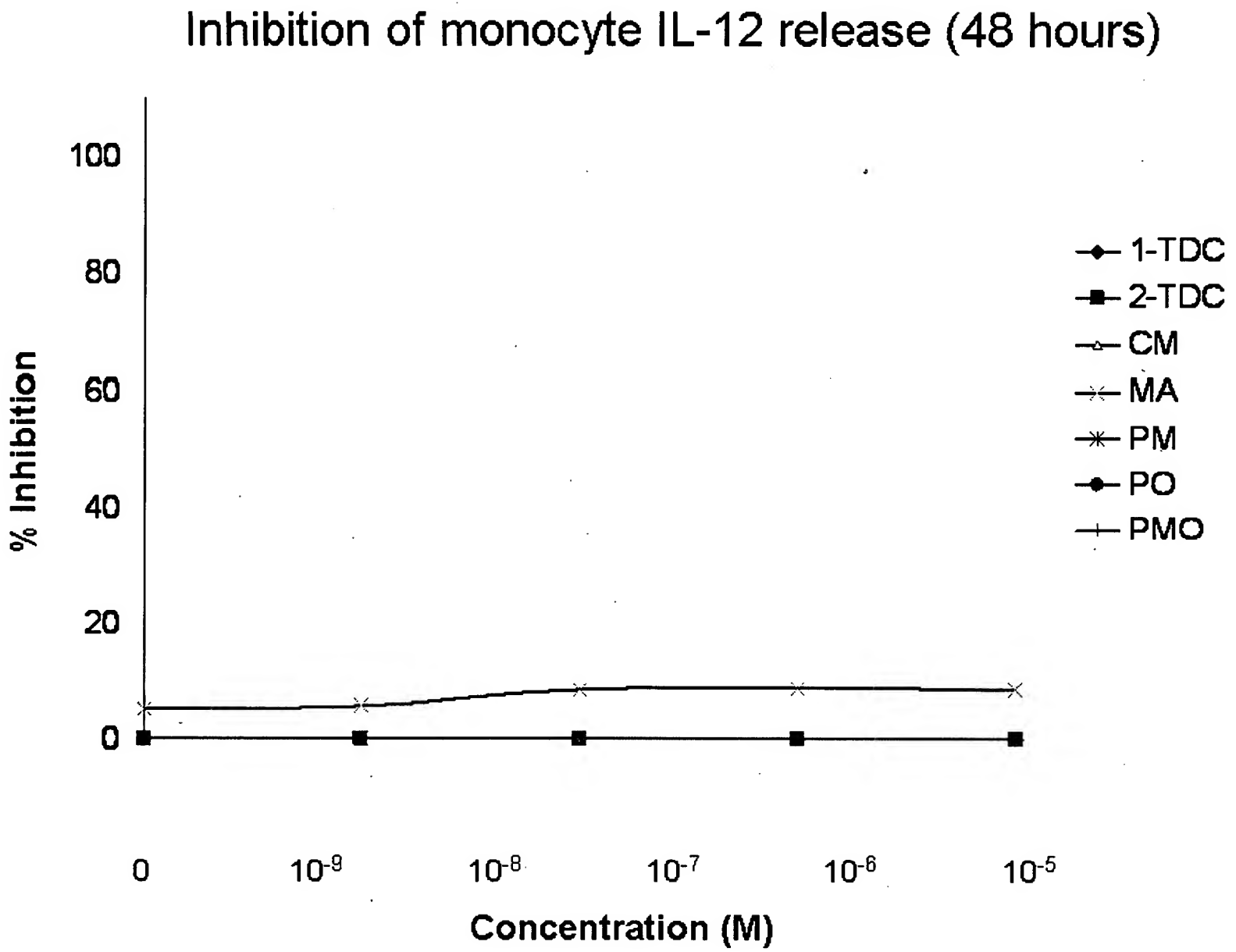

Inhibition of monocyte IL-12 release (48 hours)
% Inhibition
100
80
60
40
20
0
0
10-9
10-8
10-7
10-6
10-5
Concentration (M)
1-TDC
2-TDC
CM
MA
PM
PO
PMO

Figure 12

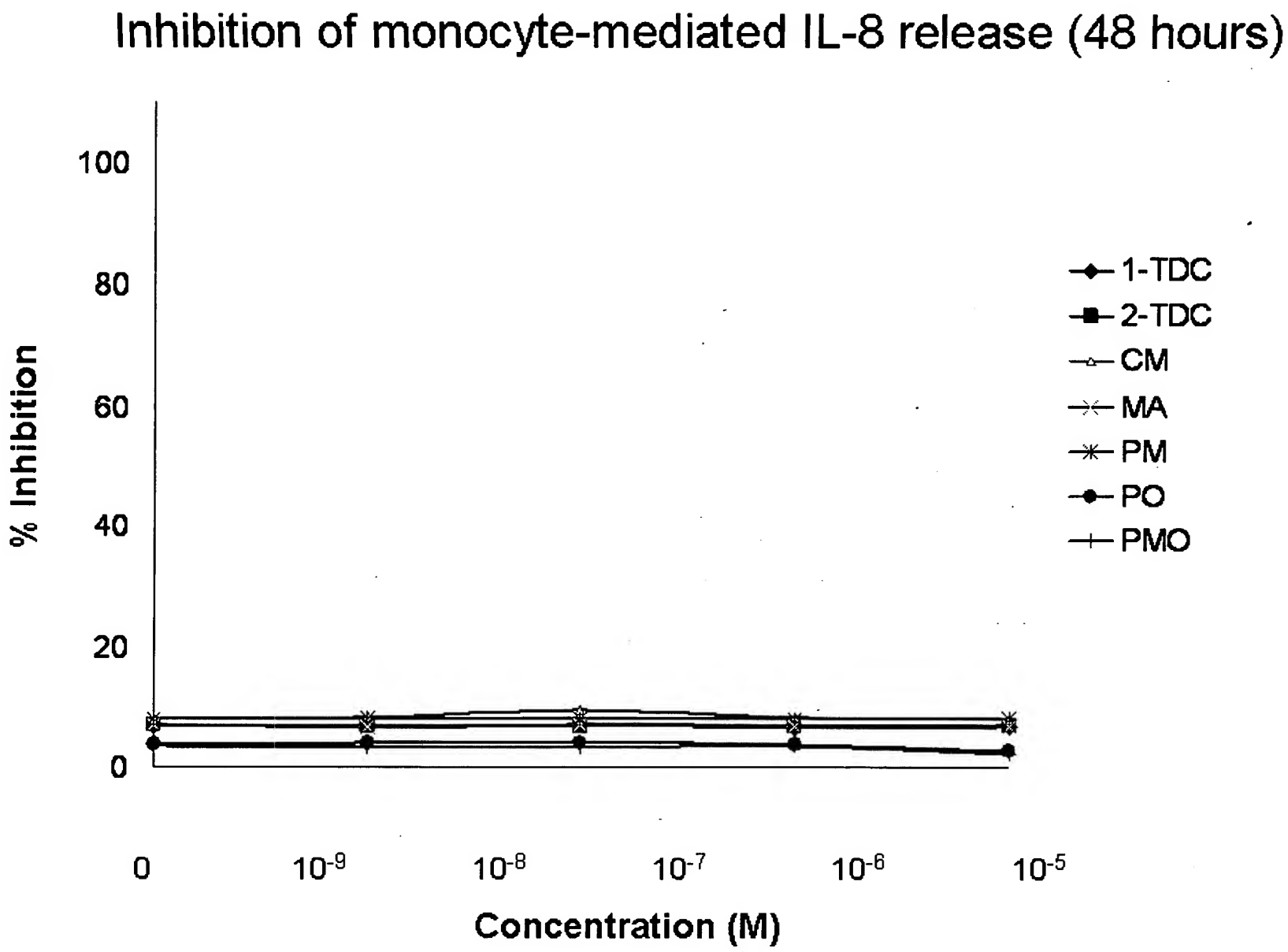
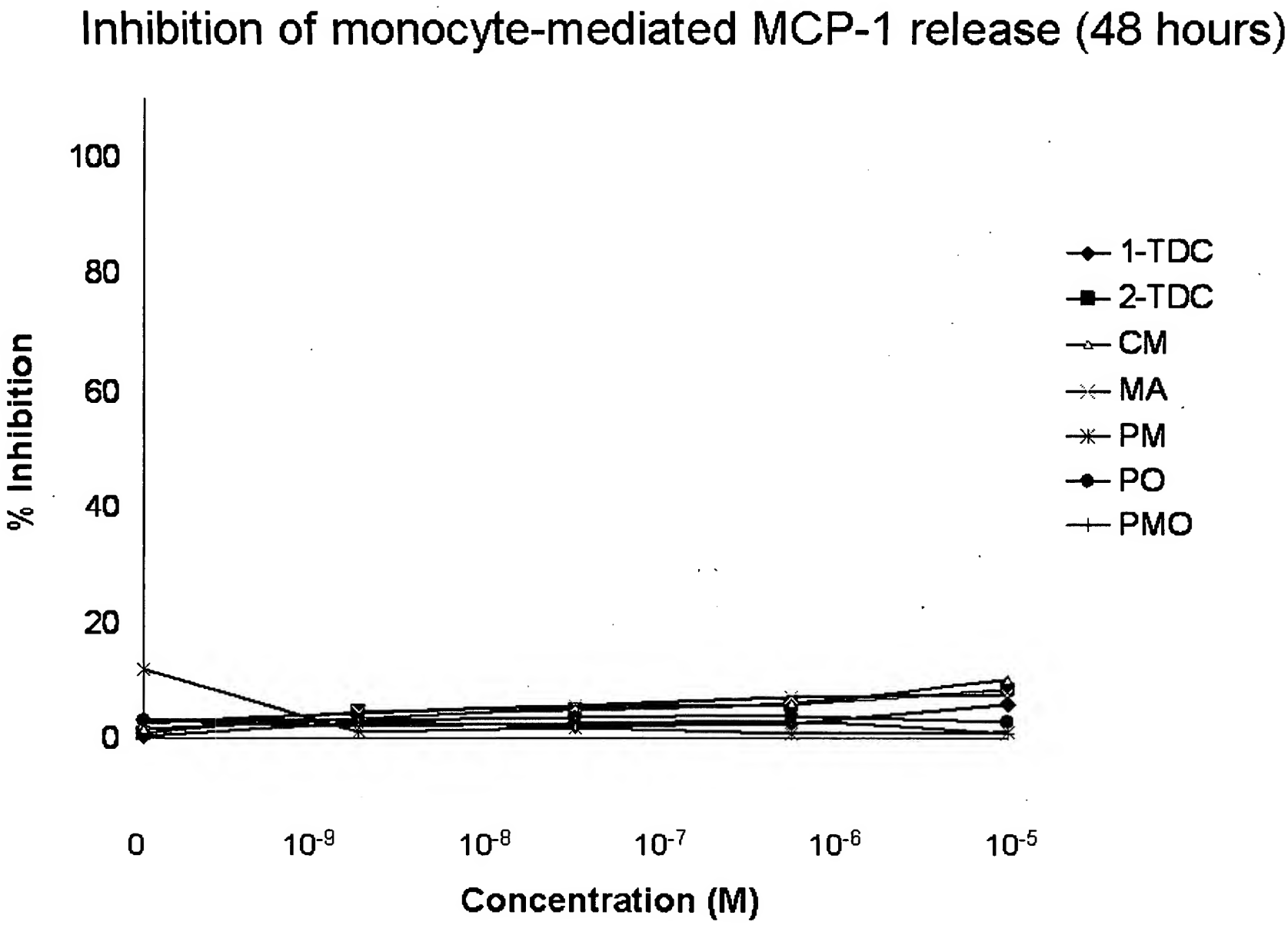

Inhibition of monocyte-mediated IL-8 release (48 hours)
% Inhibition
100
80
60
40
20
0
0
10-9
10-8
10-7
10-6
10-5
Concentration (M)
1-TDC
2-TDC
CM
MA
PM
PO
PMO

Figure 13


Inhibition of monocyte-mediated MCP-1 release (48 hours)
% Inhibition
100
80
60
40
20
0
0
10-9
10-8
10-7
10-6
10-5
Concentration (M)
1-TDC
2-TDC
CM
MA
PM
PO
PMO